

Innovative functional cAMP assay for studying G protein-coupled receptors: application to the pharmacological characterization of GPR17

Michela Buccioni · Gabriella Marucci · Diego Dal Ben · Dania Giacobbe ·
Catia Lambertucci · Laura Soverchia · Ajiroghene Thomas · Rosaria Volpini ·
Gloria Cristalli

Received: 15 June 2011 / Accepted: 20 June 2011 / Published online: 20 July 2011
© Springer Science+Business Media B.V. 2011

Abstract In this work, an innovative and non-radioactive functional cAMP assay was validated at the GPR17 receptor. This assay provides a simple and powerful new system to monitor G protein-coupled receptor activity through change in the intracellular cAMP concentration by using a mutant form of *Photinus pyralis* luciferase into which a cAMP-binding protein moiety has been inserted. Results, expressed as EC_{50} or IC_{50} values for agonists and antagonists, respectively, showed a strong correlation with those obtained with [35 S]GTP γ S binding assay, thus confirming the validity of this approach in the study of new ligands for GPR17. Moreover, this method allowed confirming that GPR17 is coupled with a $G_{\alpha i}$.

Keywords GPR17 · cAMP assay · GloSensor · Luciferine–luciferase system

Introduction

Purinergic signalling is now recognized to be involved in a wide range of activities: extracellular adenine and uracil

nucleotides mediate short-term (acute) signalling functions in neurotransmission, secretion and vasodilatation, and long-term (chronic) signalling functions in development, regeneration, proliferation and cell death [1]. In addition, P2 receptors play an important role at respiratory system and in correlated pathologies [2].

Cysteinyl leukotrienes (cys-LTs) (LTC_4 , LTD_4 , and LTE_4) are potent proinflammatory mediators which increase vascular permeability and contract smooth muscles. They are implicated in several pathologies with a clear role in respiratory diseases such as asthma and allergic rhinitis and are implicated in other inflammatory conditions including cardiovascular, gastrointestinal, skin and immune disorders [3].

Evidences suggest that among purinergic and cys-LTs signalling molecules and their receptors there are important functional interactions. The P2Y family is composed of eight receptors, which can be subdivided into two groups based on their coupling to specific G proteins. The P2Y $_1$, P2Y $_2$, P2Y $_4$, P2Y $_6$, and P2Y $_{11}$ receptors couple to $G_{\alpha q}$, to activate PLC, and the P2Y $_{12}$, P2Y $_{13}$, and P2Y $_{14}$ receptors couple to $G_{\alpha i}$, to inhibit adenylyl cyclase [4].

To date, pharmacological studies identified two receptors for cys-LTs, named CysLT1 and CysLT2, whose characteristics were then largely confirmed by molecular biology studies that allowed the cloning of two genes for the human CysLT1 and CysLT2 receptors [5].

The G protein-coupled receptor (GPCR) GPR17 is a “dual” receptor specifically activated by two unrelated families of endogenous ligands: uracil nucleotides and cys-LTs [6].

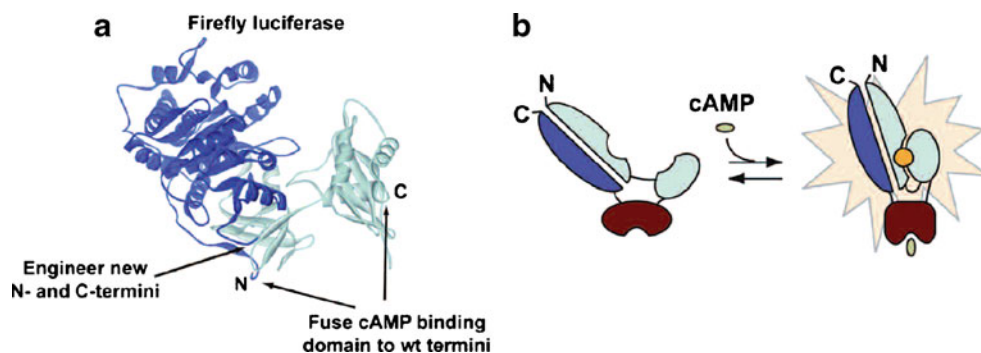
GPR17 is highly expressed in organs typically undergoing ischemic damage like the brain, heart and kidney, and its in vivo early knock down, in a rat focal brain ischemia

Michela Buccioni and Gabriella Marucci equally contributed to this work.

M. Buccioni · G. Marucci · D. Dal Ben · D. Giacobbe ·
C. Lambertucci · A. Thomas · R. Volpini · G. Cristalli (✉)
Medicinal Chemistry Unit, School of Pharmacy,
University of Camerino,
62032 Camerino, Italy
e-mail: gloria.cristalli@unicam.it

L. Soverchia
Pharmacology Unit, School of Pharmacy, University of Camerino,
62032 Camerino, Italy

Fig. 1 Overview of the GloSensor cAMP assay. **a** Schematic representation of the biosensor used in the GloSensor cAMP assay. **b** Conformational change upon binding to cAMP that produces an increase of light output



model, with either pharmacological or specific antisense strategies, reduces the progression of cerebral ischemic damage, highlighting GPR17 as novel therapeutic target for ischemia [7]. In addition, recent data have shown that GPR17 has an important role in the development and post-injury repair of damage in the brain and in the spinal cord [6]. At very early times after injury, GPR17 seems to mediate cell death and, at later stages, GPR17 may even participate in the repair mechanisms [8]. These data further highlight that this receptor could be an attractive new target for drug discovery, and the use of GPR17 selective ligands could represent a therapy strategy to contrast damage evolution in stroke.

Although a number of assay formats already exist for monitoring GPCR functions and for pharmacologically characterizing their ligands, the development of new technologies that enable increased sensitivity and of novel experimental readouts will undoubtedly advance efforts both in academic research and drug discovery.

Promega recently developed a biosensor technology called GloSensor™ cAMP assay, which provides a simple and powerful new approach to monitor GPCR activity through change in the intracellular cAMP concentration. This assay uses a mutant form of *Photinus pyralis* luciferase into which a cAMP-binding protein moiety has been inserted (Fig. 1). Upon binding of cAMP, conformational change is induced leading to increased light output

that allows evaluating the activity of ligands at the receptor under study. Following pre-equilibration with a substrate, cells stably expressing a biosensor variant can be used to evaluate GPCR function enabling easy kinetic measurements of cAMP accumulation or turnover in living cells.

Materials and methods

Cell culture

Human embryonic kidney (HEK293) L9-2 cells, as gift from Promega Corporation (Promega, Madison, WI), were grown adherently in Dulbecco's Modified Eagle Medium high glucose supplemented with 10% foetal bovine serum (FBS), 20 mM L-glutamine, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% amphotericin B and 0.2 mg/mL hygromycin and incubated at 37°C in an atmosphere of 5% CO₂, 95% O₂.

Transient transfection

Cells were seeded in a six-well plate and transfected at a confluence of about 80% by Arrest-In reagent. In particular, 3 μg of *hgpr17* and, separately, 15 μl of Arrest-In were diluted in serum-free medium. Afterwards, the DNA was

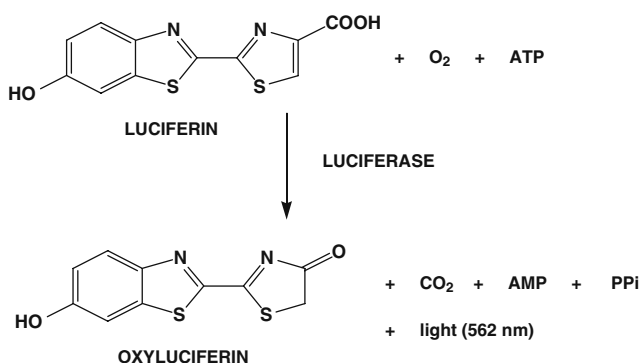


Fig. 2 Schematic representation of luciferase reaction. Oxidation of luciferin to oxyluciferin

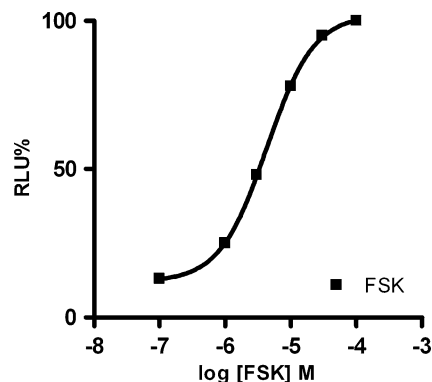


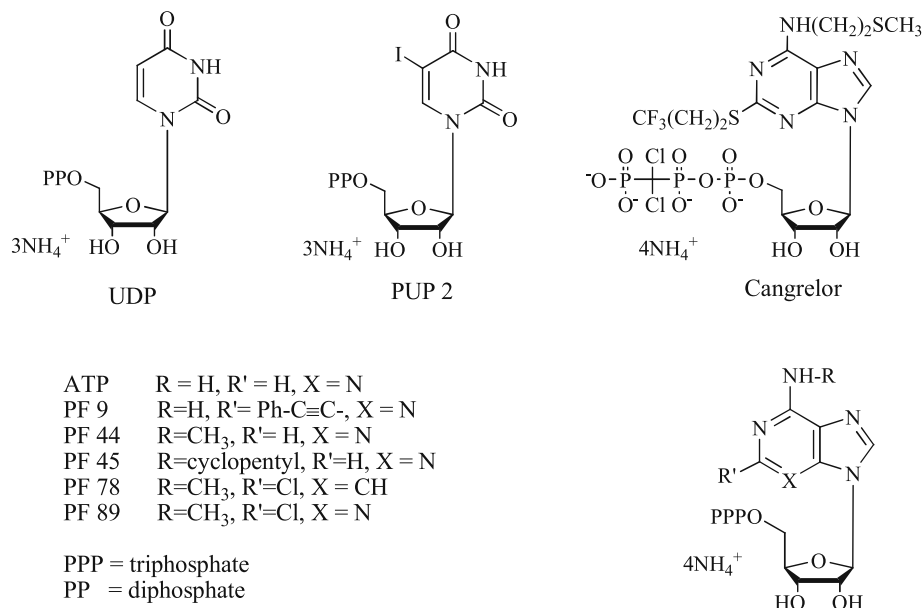
Fig. 3 FSK dose-response curve at HEK293 L9-2 cells transiently transfected with hGPR17

added to the Arrest-In reagent, mixed rapidly then incubated for 20 min at room temperature according to the manual protocol. The DNA–Arrest-In complex mixture was added into the cells and incubated for 6 h in the CO₂ incubator at 37°C [9]. After the incubation, the transfection complex was removed and fresh medium containing 10% of FBS was replaced in each well. After 48 h of incubation, the cells were used to perform a GloSensor cAMP assay.

GloSensor cAMP assay

Cells were harvested in CO₂-independent medium and were counted in a Neubauer chamber. The desiderate cell number was incubated in equilibration medium containing a 3% v/v GloSensor cAMP reagent stock solution, 10% FBS and 87% CO₂ independent medium. After 2 h of incubation, the cells were dispensed in wells of 384-well plate and, when a

Table 1 Biological activity and response of the studied compounds at HEK293 L9-2 cells transiently transfected with hGPR17 using the GloSensor cAMP assay in comparison with [³⁵S]GTPγS binding assay



Compound	Response % ^a	EC ₅₀ (nM) ^b	IC ₅₀ (nM) ^c	EC ₅₀ (nM) ^d	IC ₅₀ (nM) ^d
UDP	100	1,778 ± 320		1,140 ± 200	
UDP-galactose	100	1,008 ± 90		729 ± 42	
UDP-glucose	100	12,590 ± 680		9,500 ± 1,100	
PUP 2	100	549 ± 32		945 ± 48	
PF 9	100	0.035 ± 0.004		0.036 ± 0.003	
PF 45	100	0.18 ± 0.02		1.4 ± 0.1	
PF 78	100	0.77 ± 0.05		1.7 ± 0.1	
PF 89	100	33 ± 5		11 ± 1	
ATP	65	37 ± 4			
Cangrelor	-		0.50 ± 0.06		0.70 ± 0.02
PF 44	-		144 ± 18		112 ± 7

^a Response is expressed as percentage of the maximal relative luminescence units and is referenced against UDP, set at 100%

^b Concentration of agonist required to produce 50% of the maximum effect

^c Concentration of antagonists that produces 50% inhibition of agonist effect. Each concentration was tested five times in triplicate, and values are given as the mean ± SE

^d Data taken from [³⁵S]GTPγS binding assay [6, 11]

steady-state basal signal was obtained, agonists, at different concentrations, were added. The antagonist profile of the compounds was evaluated by assessing their ability to counteract an agonist-induced decrease of cAMP accumulation. The cells were incubated in the reaction medium (10 min at room temperature) with different antagonist concentrations and then treated with agonists. Forskolin (FSK) 10 μM was added 10 min after the agonists and various luminescence reads were performed at different incubation times.

Statistical analysis

Responses were expressed as percentage of the maximal relative luminescence units (RLU) and referred to UDP taken as 100%. Concentration–response curves were fitted by a non-linear regression using a Prism 4.0 programme (GraphPAD Software, San Diego, CA, USA). To quantify agonist potency, EC_{50} values were calculated. EC_{50} is the concentration of agonists required to produce 50% of the maximum effect. To evaluate the antagonist profile, IC_{50} values were determined. IC_{50} is the concentration of antagonists that produces 50% inhibition of the agonist effect. Each concentration was tested five times in triplicate, and the values are given as mean \pm standard error (SE).

Results and discussion

Initially, this work was focused to quantify cAMP after GPR17 stimulation by using two different immune-competitive assays, but no result was obtained. For this reason, the new sensitive and non-radioactive GloSensor cAMP assay was considered.

The assay optimization process and its validation are essential to use it as a method to screen GPR17 ligands. For

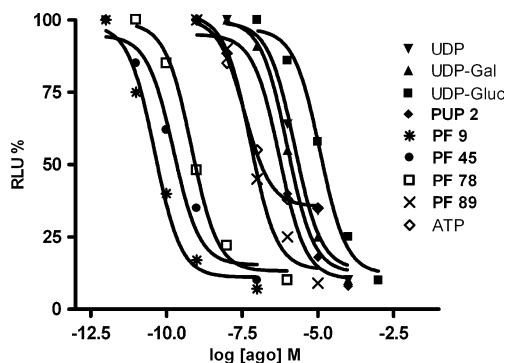


Fig. 4 Concentration–response curves in GloSensor cAMP functional assays at HEK293 L9-2 cells transiently transfected with hGPR17 of considered compounds. Each point represents the mean of five experiments performed in triplicate with a maximum SE lower than ± 10

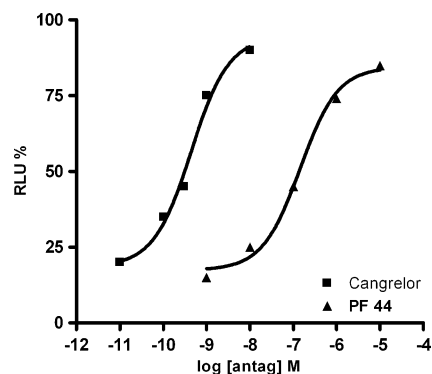


Fig. 5 Effect of Cangrelor and compound PF 44 on UDP mediated cAMP accumulation (vs forskolin, set to 100%) in HEK293 L9-2 cells transiently transfected with hGPR17. Each point represents the mean of five experiments performed in triplicate with a maximum SE lower than ± 10

this reason, at the beginning, the first step was aimed at individuating the specific crucial features of the assay that are strictly correlated to its technology itself. In particular, the number of cells, concentration of GloSensor cAMP reagent to incubate with the cells, equilibration time, and temperature were considered.

The GloSensor cAMP reagent is mainly composed of Luciferin. Luciferin is the luciferase substrate that works in the presence of cAMP produced after receptor activation. The biosensor, becoming active, interacts with its substrate luciferin converting it in oxyluciferin and emitting light at 562 nm, as reported in Fig. 2.

The equilibration time required for the GloSensor cAMP reagent will vary depending on the cell number, concentration used in the assay, and incubation temperature. During this process, the temperature should be constant, and it was noticed that at room temperature (20–25°C), the results obtained were better than those obtained at 37°C. Only when the steady-state basal signal is reached, usually after 120 min, the cells are stimulated with the agonist and luminescence measured. Generally, the luminescent kinetic of each response shows a rapid initial increase, and it remains relatively stable for 30–40 min. So, within a window of 30–40 min, luminescent signals are read. Moreover, this is a no-lytic assay that works well without the incubation with phosphodiesterase inhibitors such as rolipram suggesting the high response and system stability. The increase of cAMP accumulation by addition of UDP at HEK293 cells, stably transfected with the L9-2 plasmid expressing a genetically modified form of firefly luciferase and transiently transfected with human GPR17 (hGPR17) plasmid, was determined. HEK293 cells do not constitutively express P2Y receptors [6], and for this reason they can be utilized to characterize the hGPR17 P2Y nature.

The experiments were performed after 48 h of hGPR17 transfection utilizing UDP, UDP galactose, and UDP

glucose as referent agonists. In order to establish the concentration of FSK that produce 80% of the maximum response, the concentration–response curve was constructed (Fig. 3). Results showed that the best concentration of FSK was 10 μM . Initially, the experiments were carried out in the presence or in the absence of FSK in order to evaluate if GPR17 was coupled to G_{α_i} or G_{α_s} . Results showed that in the absence of FSK, UDP did not produce the concentration–response curve, but in the presence of it, a good concentration–response curve was obtained, confirming that this receptor is coupled with a G_{α_i} [10]. Experiments for cell number optimization were achieved. Results showed that an RLU decrease was noticed when the number of cells as well as UDP doses, compared to the control (non-stimulated cell), were increased. The number of cells necessary to get a good luminescent signal at HEK293 L9-2 cells, transiently transfected with hGPR17, was 80,000.

In order to validate this method, a series of compounds previously evaluated with [^{35}S]GTP γ S binding assay, based on the ability of agonists to increase the binding of radioactive GTP to the activated GPCR, was considered [11]. In particular, the UDP analogue PUP 2 and the ATP derivatives Cangrelor, PF 9, PF 44, PF 45, PF 78 and PF 89, differently modified in N^6 and in 2-position, were selected (see Table 1). All the chosen compounds resulted to behave as agonists in the [^{35}S]GTP γ S binding assay with the exception of Cangrelor and PF 44, which proved to be antagonists. In addition, also ATP behaviour was considered. The concentration–response curves of the tested compounds, in comparison with UDP, UDP-galactose, and UDP-glucose, are shown in Fig. 4.

The results reported in Table 1 revealed that compounds PF 9, PF 45, PF 78, PF 89, and PUP 2 are more potent than the reference agonists, with EC_{50} values very close to those obtained with the other method reported in literature [11]. In fact, the most active of the series was PF 9 with an EC_{50} value of 0.035 nM obtained with GloSensor cAMP method vs 0.036 nM reported with [^{35}S]GTP γ S binding assay. In addition, the less active compound was confirmed to be PUP 2 with an EC_{50} value of 549 vs 945 nM. Moreover, the correspondence of other compounds EC_{50} values was elevated. All agonists showed full agonist behaviour with the exception of ATP that resulted to be a partial agonist. In this cell system, no response was ever observed when cells were transfected with a corresponding empty vector.

The ability of the known purinergic antagonist Cangrelor and of PF 44 to counteract an agonist-induced decrease of cAMP accumulation in HEK293 L9-2 cells expressing the hGPR17 were also assessed. Both Cangrelor and PF 44 were able to inhibit, in a concentration-dependent manner, the decrease of cAMP induced by 50 μM UDP (Fig. 5) and by 0.5 nM PF 9 (data not shown). Results obtained with

these two agonists are very similar, confirming that they interact with the same receptor site.

A strong correlation was found by comparing these results, expressed as EC_{50} or IC_{50} values for agonists and antagonists, respectively, with the data obtained with [^{35}S]GTP γ S binding assay, thus confirming the validity of this approach in the study of new potential ligands for GPR17.

Conclusions

In this work, a new alternative assay to pharmacologically characterize new and known receptors was validated. Results obtained in terms of EC_{50} and IC_{50} values, reported and summarized in Table 1, are similar to the data reported in literature and obtained by using [^{35}S]GTP γ S binding assay. Hence, our experiments proved that this innovative and non-radioactive assay is a valid, reproducible and robust alternative to the more traditional methods, and for this reason, it may be used to screen new synthesized GPR17 ligands.

Acknowledgements This work was supported by grants from the Italian Ministry for University and Research (PRIN2008) and the Ministry for Health (Progetto Ordinario Neurolesi, RF-CNM-2007-662855).

References

- Burnstock G (2006) Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev* 58:58–86
- Wegner CD (2001) P2 receptors in the respiratory system. *Handb Exp Pharmacol* 151/II:281–300
- Capra V (2004) Molecular and functional aspects of human cysteinyl leukotriene receptors. *Pharmacol Res* 50:1–11
- Abbraccio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 58:281–341
- Mellor EA, Maekawa A, Austen KF, Boyce JA (2001) Cysteinyl leukotriene receptor 1 is also a pyrimidineric receptor and is expressed by human mast cells. *Proc Natl Acad Sci USA* 98:7964–7969
- Ciana P, Fumagalli M, Trincavelli ML, Verderio C, Rosa P, Lecca D, Ferrario S, Parravicini C, Capra V, Gelosa P, Guerrini U, Belcredito S, Cimino M, Sironi L, Tremoli E, Rovati GE, Martini C, Abbraccio MP (2006) The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. *EMBO J* 25:4615–4627
- Lecca D, Trincavelli ML, Gelosa P, Sironi L, Ciana P, Fumagalli M, Villa G, Verderio C, Grumelli C, Guerrini U, Tremoli E, Rosa P, Cuboni S, Martini C, Buffo A, Cimino M, Abbraccio MP (2008) The recently identified P2Y-like receptor GPR17 is a sensor of brain damage and a new target for brain repair. *PLoS One* 3:e3579

8. Ceruti S, Villa G, Genovese T, Mazzon E, Longhi R, Rosa P, Bramanti P, Cuzzocrea S, Abbracchio MP (2009) The P2Y-like receptor GPR17 as a sensor of damage and a new potential target in spinal cord injury. *Brain* 132:2206–2218
9. Marucci G, Lammi C, Buccioni M, Dal Ben D, Lambertucci C, Amantini C, Santoni G, Kandhavelu M, Abbracchio MP, Lecca D, Volpini R, Cristalli G (2011) Comparison and optimization of transient transfection methods at human astrocytoma cell line 1321N1. *Anal Biochem* 414:300–302
10. Benned-Jensen T, Rosenkilde MM (2010) Distinct expression and ligand-binding profiles of two constitutively active GPR17 splice variants. *BJP* 159:1092–1105
11. Calleri E, Ceruti S, Cristalli G, Martini C, Temporini C, Parravicini C, Volpini R, Daniele S, Caccialanza G, Lecca D, Lambertucci C, Trincavelli ML, Marucci G, Wainer IW, Raghino G, Fantucci P, Abbracchio MP, Massolini G (2010) Frontal affinity chromatography-mass spectrometry useful for characterization of new ligands for GPR17 receptor. *J Med Chem* 53:3489–3501